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(71) Applicant *(for all designated States except US)*: SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH [US/US]; 1275 York Avenue, New York, NY 10021 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: CORDON-CARDO, Carlos [ES/US]; Apartment 24-R, 504 East 63rd Street, New York, NY 10021 (US).

(74) Agent: WHITE, John, P.; Cooper & Dunham LLP, 1185 Avenue of the Americas, New York, NY 10036 (US).

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(54) Title: USES OF p27 IN PROSTATE CANCER

(57) Abstract

This invention provides a method for determining the aggressiveness of a prostate carcinoma comprising: (a) obtaining a sample of the prostate carcinoma; and (b) detecting the presence of p27 protein in the prostate carcinoma, the absence of p27 indicating that the prostate carcinoma is aggressive. This invention also provides a method for diagnosing a benign prostate hyperplasia comprising: (a) obtaining an appropriate sample of the hyperplasia; and (b) detecting the presence of the p27 RNA, a decrease of the p27 RNA indicating that the hyperplasia is benign. Finally this invention provides various uses of p27 in prostate cancer.

USES OF p27 IN PROSTATE CANCER

This application claims the benefit of U.S. provisional application No. 60/067,190, the contents of which is hereby incorporated by reference.

This invention was made in part with support under United States Government National Cancer Institute grant CA-DK-47650. Accordingly, the United States Government has certain rights in the invention.

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end the specification, preceding the claims.

Background of the Invention

It has been postulated that the loss of function of a new family of negative cell cycle regulators, which act as cyclin-dependent kinase inhibitors and have been termed CKI, might lead to tumor development. CKIs fall into two families, Kip and Ink, on the basis of sequence homology. $p27^{Kip1}$ is implicated in G1 phase arrest by associating with multiple G1 cyclin-dependent kinases, abrogating their activity. However, no tumor-specific $p27^{Kip1}$ genomic mutations have been found in a large group of primary human cancers studied. More recently, it has been reported that proteasome-mediated degradation of p27 protein occurs during the cell cycle and that this degradation is increased in a subset of breast and colon carcinomas of poor prognosis. **Purpose:** The present study was undertaken in order to assess for potential alterations of p27 expression in benign prostatic hyperplasia (BPH) and in a well characterized cohort of patients with prostatic cancer.

and p27 knockout animals were used. Levels of expression and microanatomical localization of p27 protein and RNA transcripts were determined by immunohistochemistry and *in situ* hybridization with specific antibodies and probes, respectively. Comparative analyses between immunohistochemistry, immunoblotting and immunodepletion assays were also conducted in a subset of cases. Association between alterations in p27 expression and clinicopathological variables were evaluated using the two-tailed Fisher's exact test. Disease relapse-free survivals were evaluated using the Kaplan-Meier method and the Logrank test. Distinct anomalies in the expression of p27 in benign and malignant human prostate tissues are reported. The normal human prostate shows abundant amounts of p27 and high levels of p27 messenger in both epithelial and stroma cells. However, p27 protein and transcripts are almost undetectable in epithelial and stroma cells of BPH lesions. It is also reported that p27-null mice develop hypercellular prostatic glands which histologically resemble human BPH. Based on these findings we postulate that the loss of p27 expression in human prostate may be causally linked to BPH. Prostatic carcinomas can be categorized into two groups: those that contain detectable p27 protein and those that do not. In contrast to BPH, however, both groups of prostatic carcinomas contain abundant p27 transcripts. Moreover, primary prostatic carcinomas displaying the p27-negative phenotype appear to be biologically more aggressive, based on their association with time to prostate specific antigen (PSA) failure following radical prostatectomy. These results support the postulate that BPH is not a premalignant lesion in the pathway of prostate cancer development. Data also suggest that prostatic carcinoma develops along two different pathways, one involving the loss of p27 and the other using other processes that circumvent the growth suppressive effects of p27.

The vector includes, but is not limited to, an adenovirus vector, adeno-associated virus vector, Epstein-Barr virus vector, Herpes virus vector, attenuated HIV virus, retrovirus vector and vaccinia virus vector.

This invention provides a method for prolong life-span of patient with prostate carcinoma which comprises introducing an effective amount of p27 protein into the carcinoma cell so as to thereby prolong the life-span of the patient with said prostate carcinoma.

This invention provides a method for prolong life-span of patient with prostate carcinoma which comprises introducing an effective amount of a substance capable of stabilizing the p27 protein into the carcinoma cell so as to thereby prolong the life-span of the patient with said prostate carcinoma.

This invention provides a composition for prolong life-span of patient with prostate carcinoma which comprises an effective amount of a nucleic acid molecule having sequence encoding a p27 protein and a suitable carrier.

This invention provides a composition for prolong life-span of patient with prostate carcinoma which comprises an effective amount of the p27 protein and a suitable carrier.

This invention provides a composition for prolong life-span of patient with prostate carcinoma which comprises an effective amount a substance capable of stabilizing the p27 protein and a suitable carrier.

trough (F) 400x.

Figure 2. In certain prostatic carcinomas p27 protein is a functional cyclin-dependent kinase inhibitor. (A) Immunohistochemical staining correlates with the presence of p27 by immunoblotting. Tumors #1 and #2 were negative and tumor #3 positive for p27 protein expression, paralleling their IHC patterns. (B) Immunodepletion of p27 extracts. Extracts obtained from tumors #2 and #3 were subjected to sequential depletion with antibodies specific to p27 or a non-specific rabbit anti-mouse (RaM). Following depletion, the proteins in the supernatants were resolved and the presence of p27 determined by immunoblotting. (C) Depletion of p27 depletes heat stable cyclin-dependent kinase inhibitory activity. The supernatant shown in panel B was boiled and following clarification the soluble fraction was incubated with different amounts of recombinant cyclin E/CDK2 kinase and the degree of inhibition of cylin E/CDK2 activity on histone H1 substrate was measured. The amount of each kinase used is shown in the panel and the bars are representative activities on an arbitrary scale. Depletion with either RaM or p27 specific antibodies did not affect the inhibitory activity of the p27 negative tumor; however, depletion of p27 from the positive tumor extract completely ablated the heat stable inhibitor activity.

Figure 3. Recurrence-free proportion analysis of patients with primary prostate carcinoma (n=42) as assessed by time to detectable PSA. Patients who had PSA relapse were classified as failures, and patients with PSA relapse, or those who were still alive or died from other disease or lost to follow-up during the study period, were coded as censored. Time to relapse was defined as the time from date of surgery to the endpoint (relapse or censoring). Disease relapse-free survivals were evaluated using the Kaplan-Meier method and the Logrank test. A trend was observed between a p27 negative phenotype and early relapse

staining of a prostate gland of a p27+/+ mouse showing well defined acini of epithelial cells surrounded by a stroma containing few fibroblasts and poor in supportive connective tissue components. (B) Hematoxylin and eosin staining of a prostate gland of a p27-/- mouse showing multiple and complex glands and hypercellular acini of epithelial cells surrounded by fibromuscular stroma cells in a connective tissue displaying abundant supportive components. (C and D) Hematoxylin and eosin stainings of a prostate gland of a p27-/- mouse, high power details, illustrating the complexity of the glands and abundant fibromuscular stroma elements (C), as well as the hypercellularity of the acini (D). Original magnifications: (A) and (B) 200x; (C) and (D) 400x.

embodiment, the nucleic acid molecule comprises a vector. The vector includes, but is not limited to, an adenovirus vector, adeno-associated virus vector, Epstein-Barr virus vector, Herpes virus vector, attenuated HIV virus, retrovirus vector and vaccinia virus vector.

Methods to introduce a nucleic acid molecule into cells have been well known in the art. Naked nucleic acid molecule may be introduced into the cell by direct transformation. Alternatively, the nucleic acid molecule may be embedded in liposomes. Accordingly, this invention provides the above methods wherein the nucleic acid is introduced into the cells by naked DNA technology, adenovirus vector, adeno-associated virus vector, Epstein-Barr virus vector, Herpes virus vector, attenuated HIV vector, retroviral vectors, vaccinia virus vector, liposomes, antibody-coated liposomes, mechanical or electrical means. The above recited methods are merely served as examples for feasible means of introduction of the nucleic acid into cells. Other methods known may be also be used in this invention.

This invention provides a method for prolong life-span of patient with prostate carcinoma which comprises introducing an effective amount of p27 protein into the carcinoma cell so as to thereby prolong the life-span of the patient with said prostate carcinoma.

This invention provides a method for prolong life-span of patient with prostate carcinoma which comprises introducing an effective amount of a substance capable of stabilizing the p27 protein into the carcinoma cell so as to thereby prolong the life-span of the patient with said prostate carcinoma. Such substance may be either inhibiting the protease which degrade the p27 protein or it may interact with p27 in such a way that the protein will be resistant to degradation. By administering such substance into the cell, the effective amount of p27 protein will be

Experimental Details

MATERIALS AND METHODS

Patient Characteristics and Tissues. A cohort of 74 patients with prostatic carcinoma were evaluated. Tissues were obtained from the Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York. Samples were formalin-fixed, paraffin-embedded tissue specimens. Fourty-two primary prostate adenocarcinoma specimens were evaluated, as well as 9 metastases to lymph node and 23 metastases to bone. Normal prostatic tissue and/or areas of benign prostatic hyperplasia adjacent to tumor were observed in the majority of the primary cases studied. These tissues were also analyzed as part of the study. In addition, 10 pairs of frozen normal and tumor prostate tissues were utilized for antibody titration, as well as comparative analyses between immunohistochemistry, immunoblotting and immunodepletion assays (see below). Representative hematoxylin-eosin stained sections were examined to evaluate the histopathological characteristics of the lesions to be analyzed, including the ratio of normal-to-tumor content for microdissection techniques.

In order to evaluate prostatic tissue of p27 null mice, eight 7 month old and six greater than 12 month old littermate pairs of wild-type and p27 knockout animals were used. Tissues were dissected, weighted and processed for histology by formalin fixation and paraffin embedding. Tissue sections were cutted and stained with hematoxylin-eosin for histologic analysis. All sections were utilized to count the number of acini per gland, a process that was conducted utilizing magnifications of 200x.

Antibodies and Immunohistochemistry. The following well characterized antibodies and corresponding final working dilutions were used for the present study: monoclonal antibody p27/Kip1 (Ab-2, Oncogene Science, Boston, MA - 0.1

sections were rinsed in water and PBS for 10 minutes. The slides were digested with Proteinase K (50ug/ml) for 18 minutes at 37°C in PBS, and post-fixed at 4 °C in a freshly prepared solution of 4% paraformaldehyde in PBS for 5 minutes. Prehybridization was done for 30 minutes at 45 °C in 50% formamide and 2XSSC. The hybridization buffer consisted of 50% deionized formamide (v/v), 10% dextran sulphate (50% stock solution), 2XSSC (20X stock solution), 1% SDS (10% stock solution), and 0.25 mg/ml of herring sperm DNA (10 mg/ml). Hybridization was peformed overnight at 45 °C applying 10 pmol/L digoxigenin-labeled riboprobe in 50 ul of hybridization buffer per section under a coverslip. The coverslips were removed and the slides were washed in pre-warmed 2XSSC for 20 minutes at 60 °C twice, followed by washes in pre-warmed 0.5XSSC and 0.01XSSC at 60 °C for 20 minutes, respectively. After these washes the slides were incubated in normal sheep serum diluted in buffer pH 7.5 and successively in the same buffer with antibody anti-digoxigenin-AP (Boehringer Mannheim, Indianapolis, IN) at dilution of 1:1500 for 1 hour at room temperature. The visualization was accomplished by nitro-blue tetrazolium 5-bromo-4-chloro-3-indoylphosphate. The slides were counterstained with methyl green and mounted.

Immunoblotting and Immunodepletion Assays. Proteins were extracted from three OCT-embedded prostatic carcinomas and resolved on polyacrylamide gels for immunoblotting with p27-specific antibodies. Extracts obtained from p27 positive and negative tumors were subjected to sequential depletion with antibodies specific to p27 or a non-specific rabbit anti-mouse (RaM). Following depletion, the proteins in the supernatants were resolved and the presence of p27 determined by immunoblotting. Aliquots of these supernatants were briefly boiled and following clarification the soluble fraction was incubated with different amounts of recombinant cyclin E/CDK2 kinase and the degree of inhibition of cylin E/CDK2 activity on

EXPERIMENTAL RESULTS AND DISCUSSION

To determine whether loss of p27 expression was a common feature in prostate cancer, we analyzed 74 prostate carcinomas from primary and metastatic sites, representing different hormone sensitivities. Included were 42 hormone-naïve primary tumors, some with associated prostatic intraepithelial neoplastic (PIN) lesions, and 32 metastatic carcinomas from lymph node tumors (n=9) and bone metastases (n=23). Thirteen of these metastatic lesions were from hormone-naïve cases, while the remaining 19 metastases were obtained after hormonal treatment. PIN lesions displaying a cribiform or pseudopapillary pattern expressed high levels of p27 protein (Figure 1A) and were associated with p27-positive invasive prostatic carcinomas. In contrast, PIN lesions displaying a flat growth pattern had low to undetectable p27 levels (Figure 1B) and were associated with p27-negative invasive tumors. Of the invasive primary prostatic carcinomas studied, 12 of 42 (28.5%) cases had an intense nuclear immunoreactive p27 pattern in the malignant cells (data not shown). The remaining 30 (71.5%) primary neoplasms displayed altered patterns of expression: 12 cases had undetectable p27 levels (Figure 1C), while 18 cases had a heterogeneous pattern of expression (data not shown). In metastatic lesions, 7 of 32 (21.9%) showed intense p27 nuclear immunostaining in most tumor cells (Figure 1D). The remaining 25 (78.1%) metastatic lesions had either heterogeneous (data not shown) or undetectable nuclear expression of p27 (Figures 1E and 1F). Interestingly, all but one of the nine patients with hormone-independent bone lesions displayed altered p27 expression. Four of these 9 cases had undetectable p27 protein expression (Figure 1F), 4 cases had heterogeneous patterns of p27 expression ranging from 30% to 40% tumor cells with weak positive staining, and one case displayed 80% positive tumor cells. However, high levels of $p27^{Kip1}$ mRNA, as determined by in situ hybridization to a p27 cDNA probe, were found in all

complete, were considered. A trend toward an association was observed between a p27 negative phenotype and early relapse (p=0.08) (Figure 3). This difference did not reach statistical significance due to the limited sample size of the cohort analyzed. Supporting this concept is the fact that in a multivariate proportional hazards analysis, after controlling for stage and Gleason score, p27 status still was the strongest factor in predicting PSA relapse (p=0.07).

These data suggest extending the characterization of p27 expression to normal prostate and benign prostatic hyperplasia. In the normal human prostate, abundant amounts of p27 protein were detected in the ductal and acinar cells, mainly luminal elements, as well as stroma cells using immunohistochemistry. Epithelial cells displayed a strong nuclear immunostaining signal (Figure 4A). Likewise, both epithelial and stroma cells expressed abundant p27 transcripts (Figures 4B and 4C), as detected by in situ hybridization. Strikingly, in 12 cases of BPH p27 expression was low to undetectable in epithelial and stroma cells in the hyperplastic nodules. Immunohistochemical staining revealed low to undetectable immunoreactivities in both epithelial and fibromuscular cells in the hyperplastic nodules (Figure 4D). This contrasts with the strong p27 nuclear immunostaining phenotype observed in the normal prostate. Likewise, p27 mRNA transcript levels were low to undetectable on consecutive sections of BPH by in situ hybridization (Figure 4E and 4F). In some of these BPH tissue samples we found areas of basal cell hyperplasia. These cellular elements also had low to undetectable amounts of p27 protein and transcripts (data not shown). Nevertheless, in the non-hyperplastic regions of these same BPH samples, normal ductal and acinar epithelial cells, as well as stroma elements, showed high levels of p27 expression. These results indicate that in the development of BPH, p27 transcription may be down-regulated. This finding was

was associated with histopathological differences that became more accentuated in the elderly group. The hyperplastic prostate of the older p27-/- mice showed enlarged glands, development of hypercellular acini of epithelial cells, and an increase in fibromuscular stroma cells (Figure 5). These histological changes are reminiscent of BPH in humans and support the hypothesis that the loss of p27 expression in human prostate may be causally linked to BPH.

It has been suggested that BPH and malignant prostate growth share a common pathway because they commonly coexist and demonstrate androgen dependency (42-44). However, this relationship remains unclear since BPH tends to develop in the transition zone, while the majority of carcinomas develop in the peripheral zone (45-48). Results from the present study reveal that, unlike in the BPH lesions, prostatic carcinoma cells regulate p27 expression at the post-transcriptional level. Taken together these data support the postulate that BPH is not a premalignant lesion in prostate cancer development.

Coordinate inactivation of the pathways involving the p53 and RB genes appears to be an essential requirement for the genesis of most human cancers. However, both p53 mutations and RB alterations are reported to be late and uncommon events in prostate tumor progression (49-52). Contrary to these results, data from this study indicate that inactivation of p27 is a frequent and early event in some prostate cancers. It is thus our working hypothesis that p27 represents another pathway of tumor suppression in certain human tumors, prostate cancer being a paradigm in which this concept could be further tested.

In summary, data from this study suggest that $p27^{Kip1}$ gene ablation in the mouse causes a pronounced prostatic hyperplasia, and that the loss of p27 expression in human prostate may be causally linked to BPH. In addition, data

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protein into the carcinoma cell under conditions permitting expression of said gene so as to prolong the life-span of the patient with said prostate carcinoma.

7. The method of claim 6, wherein the nucleic acid molecule comprises a vector.

8. The method of claim 7, wherein the vector is an adenovirus vector, adenoassociated virus vector, Epstein-Barr virus vector, retrovirus vector or vaccinia virus vector.

9. A method for prolong life-span of patient with prostate carcinoma which comprises introducing an effective amount of p27 protein into the carcinoma cell so as to thereby prolong the life-span of the patient with said prostate carcinoma.

10. A method for prolong life-span of patient with prostate carcinoma which comprises introducing an effective amount of a substance capable of stabilizing the p27 protein into the carcinoma cell so as to thereby prolong the life-span of the patient with said prostate carcinoma.

11. A composition for prolong life-span of patient with prostate carcinoma which comprises an effective amount of a nucleic acid molecule having sequence encoding a p27 protein and a suitable carrier.

12. A composition for prolong life-span of patient with prostate carcinoma which comprises an effective amount of the p27 protein and a suitable carrier.

13. A composition for prolong life-span of patient with prostate carcinoma which comprises an effective amount a substance capable of stabilizing the p27 protein and a suitable carrier.

FIG. 1C

FIG. 1F

FIG. 1B

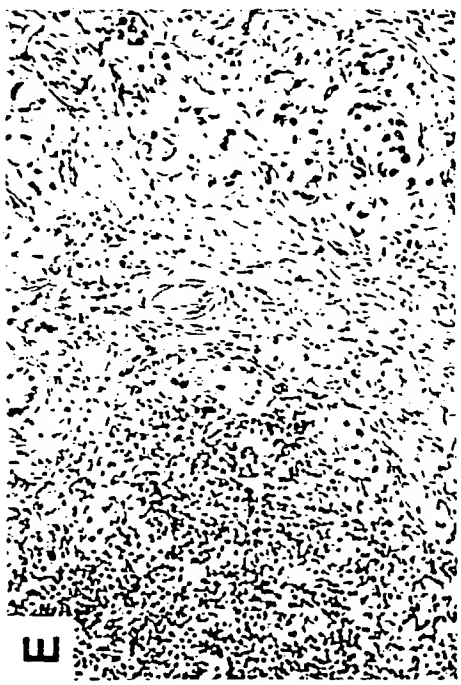


FIG. 1E

FIG. 1A

FIG. 1D

FIG. 1H

FIG. 1G

FIG. 2A

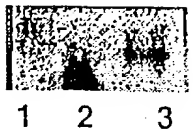


FIG. 2B

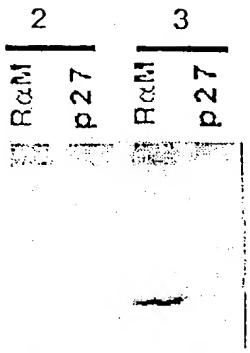


FIG. 2C

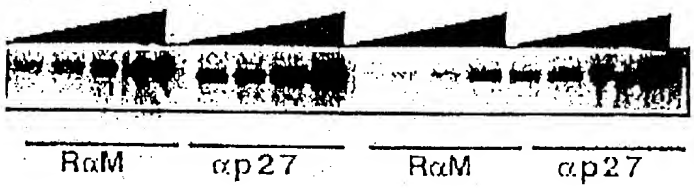
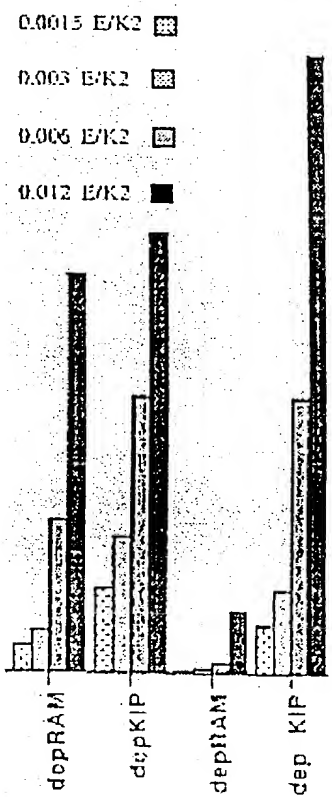
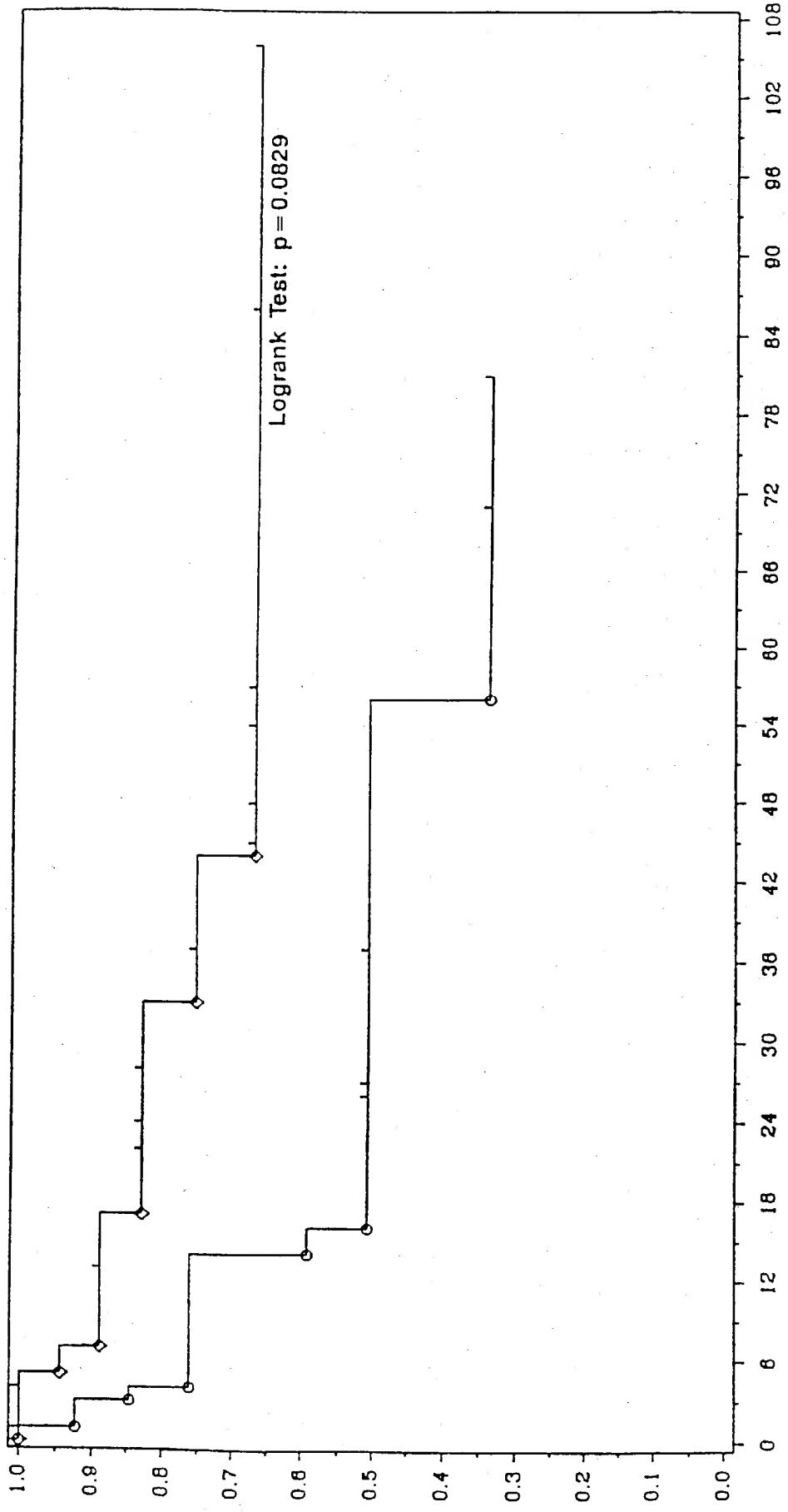


FIG. 2D

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RECURRENCE-FREE PROPORTION
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0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
6
12
18
24
30
36
42
48
54
60
66
72
78
84
90
96
102
108
MONTHS
p27 <20% (14 Pts. 6 Censored)
p27 >=20% (28 Pts. 16 Censored)
ick mark(|) Indicates last follow-up

FIG. 3

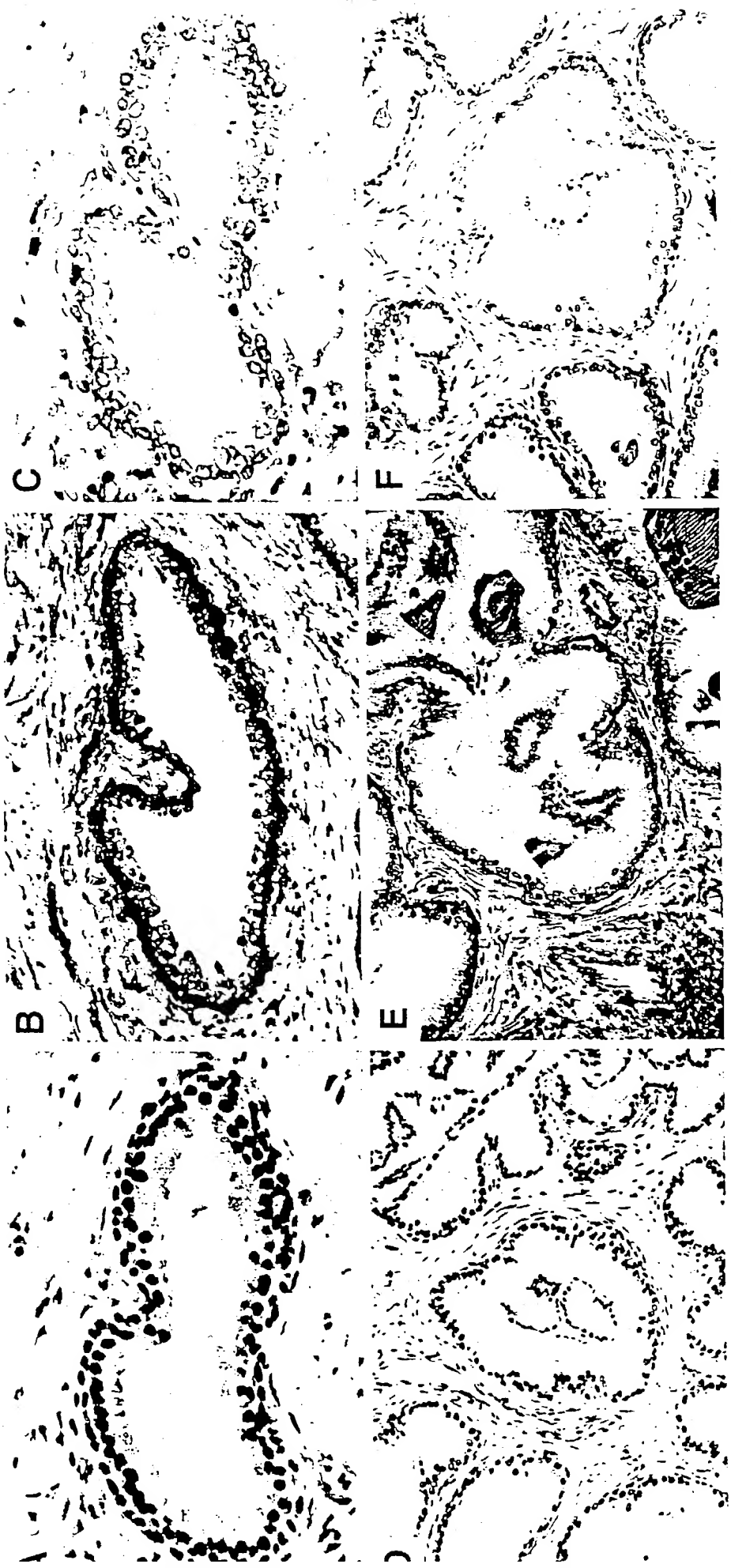

FIG. 4A

FIG. 4B

FIG. 4C

FIG. 4D

FIG. 4E

FIG. 4F

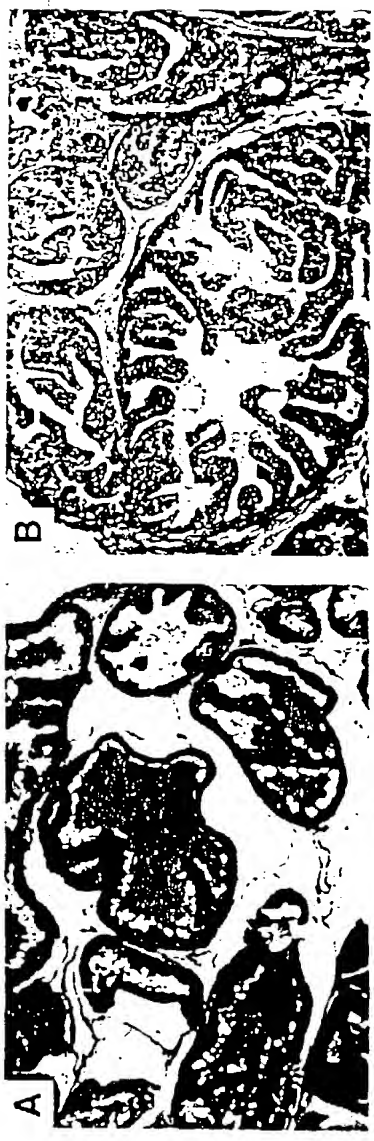

FIG. 5A

FIG. 5B

FIG. 5C

FIG. 5D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/25483

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 6; 424/138.1; 514/2, 44; 536/23.5, 23.2; 530/300, 350, 387.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CAPLUS, EMBASE, GENBANK
search terms: p27kip1, ?kip1, p27, cyclin, prostate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	US 5,688,665 A (MASSAGUE et al.) 18 November 1997, figure 7c; figure 12; column 52, lines 12-13; abstract. column 3, lines 59-65 and column 4, lines 14-26.	3,4,6,7,9-13 --- 5,8
X	HENGST, et al. Translational control of p27Kip1 accumulation during the cell cycle. Science. 29 March 1996, Vol. 271, pages 1861-1864, especially page 1861 and figures 1 and 2.	1-4, 11-13

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 18 FEBRUARY 1999	Date of mailing of the international search report 02 MAR 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NANCY A. JOHNSON Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/25483

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

G01N 33/53, 33/68; C12Q 1/168; A61K 31/00, 39/395, 38/02, 48/00; C07H 21/04; C07K 14/47, 16/18, 16/40

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/7.1, 6; 424/138.1; 514/2, 44; 536/23.5, 23.2; 530/300, 350, 387.7